On the Generic Position of Polyommatus avinovi (Lepidoptera: Lycaenidae)*

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Polyommatus avinovi (Stshetkin, 1980), an enigmatic taxon from Tajikistan has been considered in the literature either as a member of the genus *Polyommatus*, or a taxon belonging to the genus *Rimisia*. None of the conclusions on taxonomy and nomenclature of *P. avinovi* were supported by molecular or cytological data, therefore the problem of identity and phylogenetic position of this taxon has remained unsolved. Here we use the barcoding fragment of the *COI* gene as a molecular marker to demonstrate that none of these hypotheses are true. Phylogenetic analysis revealed *P. avinovi* to be strongly differentiated from both *Polyommatus* and *Rimisia*. Instead, it formed a separated, well supported monophyletic clade within the genus *Afarsia* Korb & Bolshakov, 2011. Thus, we propose the following new combinations for this butterfly: *Afarsia avinovi* comb. nov. and *Afarsia avinovi dangara* comb. nov.

Key words: Lepidoptera, Lycaenidae, Polyommatina, phylogeny, *Afarsia avinovi* comb. nov., *Afarsia avinovi dangara* comb. nov., DNA barcode.

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Despite current progress in studies of blue butterflies belonging to the subtribe Polyommatina (e.g. LUKHTANOV *et al.* 2008, 2014, 2015; VODO-LAZHSKY & STRADOMSKY 2010; WIEMERS *et al.* 2010; TALAVERA *et al.* 2013a, 2013b; SANUDO-RESTREPO *et al.* 2013; PRZYBYŁOWICZ *et al.* 2014; STRADOMSKY 2014; SHAPOVAL & LUKHTANOV 2015a, 2015b; ECKWEILER & BOZANO 2016), a large number of unresolved taxonomic problems still persist in this group. Among them, the taxonomic position of the enigmatic species *Polyommatus avinovi* Stshetkin, 1980 is one of the most challenging questions.

This taxon was described from Tajikistan (Peter the Great mountain range) on the basis of several exemplars with a remarkable and easily distinguishable pattern remarkable and easily distinguishable wing pattern (STSHETKIN 1980) (Fig. 1a). At a later time, a remote population from Dangara (Tajikistan) was discovered and described as a subspecies of *Polyommatus avinovi*, named *Polyommatus (Agrodiaetus) avinovi dangara* (Eckweiler, 1997) (Fig. 1b). According to the original description, *P*. (*A*.) *avinovi dangara* differs slightly in size and phenotype from *P*. (*A*.) *avinovi avinovi*. A similar hind wing underside pattern, characterized by exaggerated spots and arcuate alveoles, bears another Tajik species – *Polyommatus (Agrodiaetus) magnificus* (Grum-Grshimailo, 1885) (= *superbus* Staudinger, 1887), but it differs from *Polyommatus avinovi* in having blue scales on the forewing upperside (Fig. 1c). Morphological similarity led to suggestion, that *Polyommatus magnificus* and *Polyommatus avinovi* were closely related species.

Recently, P. (A.) avinovi avinovi and P. (A.) avinovi dangara, treated by Eckweiler as a members of the subgenus Polyommatus (Agrodiaetus), were placed by Zhdanko, based on the male genitalia morphology, to the genus Rimisia (ZHDANKO 2004), close to Rimisia miris (Fig. 1d). It should be noted that analyses based mainly on external morphological characters alone did not always clarify the phylogenetic positions and identities of many lycaenid taxa, especially those belonging to the subgenus

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Fig. 1. Upperside (left) and underside (right) of Afarsia avinovi, Polyommatus magnificus, Afarsia sieversi haberhaueri and Rimisia miris. See main text for explanations of the spot morphology, indicated by arrows. a – A. avinovi avinovi, female (Tajikistan, Gissar Range, Karatak river, Shurkok village, 1500 m, 23.VII.2004, GenBank accession number KY084480); b – A. avinovi dangara, male (Tajikistan, Pamir/Alat, Dangara, 16.VI.1991, GenBank accession number KY084479); c – Polyommatus magnificus, male (Alai mnts Kitschi-Karamuk, type specimen of Lycaena magnifica Grum-Grshimailo, 1885 from collection of the Natural History Museum, London, Specimen ID: BMNH # 264665); d – Rimisia miris, male (Kazakhstan, near Balkhash, 02.VI.1909, from collection of the Zoological Institute RAS); e – Afarsia sieversi haberhaueri, male (10.VI.1938, Uzbekistan, Samarkand, Zaravshan, from collection of the Zoological Institute RAS).

Agrodiaetus (TALAVERA et al. 2013a; LUKHTANOV et al. 2014). A DNA-based phylogenetic study confirmed placement of P. (A.) magnificus within the subgenus Agrodiaetus (genus Polyommatus) (KANDUL et al. 2007). Unlike the abovementioned taxon, P. (A.) avinovi has never been studied genetically, therefore its identification, taxonomic status and phylogenetic position have remained unverified. Our paper provides the first molecular analysis of this taxon.

Material and Methods

Two specimens (*Afarsia avinovi avinovi*, Gen-Bank accession number KY084480 and *Afarsia avinovi dangara*, GenBank accession number KY084479) were processed at the Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences. DNA extraction from a single leg removed from each specimen was done using the QIAamp DNA Investigator Kit (Qiagen, Netherlands) according to the manufacturer's protocol.

Standard lepidopteran barcode primers (HEBERT *et al.* 2004) failed to amplify a sufficient product. Thus, two self-designed forward primers (Nz COI b - TAC AAT TTA TCG CTT ATA AAC TCA; DRD4F - TAG AAA ATG GAG CAG GAA) and two reverse primers (MH-MR1 (LUKH-TANOV *et al.* 2009), Nancy (FOLMER *et al.* 1994) were used for DNA amplification and resulted in a 671 bp fragment of the mitochondrial *cytochrome oxidase I* gene (*COI*).

The PCR amplifications were performed in a 50 μ l reaction volume containing ca. 10-20 ng genomic DNA and 0.5 mM each of forward and reverse primer, 1 mM dNTPs, 10x PCR Buffer (0.01 mM Tris-HCl, 0.05 M KCl, 0.1% Triton X-100: pH 9.0), 1 unit Taq DNA Polymerase (Thermo Fisher Scientific, Lithuania), 5 mM MgCl₂. The temperature profile was as follows: initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Amplified fragments were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania). Purification was carried out according to the manufacturer's protocol. The success of PCR amplification and purification was evaluated by electrophoresis of the products in 1% agarose gel. Purified PCR product was used for direct sequencing. Sequencing of the doublestranded product was carried out at the Research Resource Center for Molecular and Cell Technologies (St. Petersburg State University).

Seven specimens (Afarsia morgiana badgiri HM404831-HM404835 and Afarsia morgiana se*leucus* HM404846-HM404847) were processed at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using standard high-throughput protocol described in DEWAARD *et al.* (2008) and resulted in a 658 bp fragment of *COI*. The set of voucher specimens of the butterflies is kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg) and at the McGuire Center for Lepidoptera and Biodiversity (University of Florida).

Representatives of all principal genera of the Polyommatina subtribe (= *Polyommatus* section sensu Eliot) available from the GenBank database (WIEMERS & FIEDLER 2007; LUKHTANOV et al. 2009; TALAVERA et al. 2013a) were included in our analysis to clarify the generic position and phylogenetic relationships of P. avinovi avinovi and P. avinovi dangara. Since Leptotina and Cupidina subtribes were earlier inferred to be the sisters groups to the Polyommatina subtribe (TALAVERA et al. 2013a), we use members of these subtribes to root the phylogram. A complete list of specimens included in this study is given in Table 1. A Bayesian approach, maximum-likelihood (ML) and maximum-parsimony (MP) analyses were used for estimating the phylogeny. Bayesian analysis was performed using the program MrBayes 3.1.2 with the nucleotide substitution model GTR+G+I. jModelTest was used to determine optimal substitution models for Bayesian inference (BI) analysis (POSADA 2008). TRACER, version 1.4 was used for summarizing the results of the Bayesian phylogenetic analysis (http://beast.bio.ed.ac.uk/Tracer).

Maximum-parsimony (MP) and maximumlikelihood (ML) analyses were performed using MEGA5 software (TAMURA *et al.* 2011). For MP analysis a heuristic search was carried out using the close-neighbor-interchange algorithm (NEI & KUMAR 2000). One hundred bootstrap pseudoreplicates were obtained under a heuristic search with the random addition of sequences, saving no more than ten equally parsimonious trees per replicate. We used non-parametric bootstrap values (FEL-SENSTEIN 1985) to estimate branch support on the reconstructed ML and MP trees. Branch supports were inferred from 1000 bootstrap replicates.

Results and Discussion

Phylogenetic analysis of the gene *COI* resulted in a consensus phylogram which mostly displayed a high level of posterior probability and bootstrap values for the majority of the genus-level clades. At the same time the evolutionary relationships between some genera remained unresolved (Fig. 2).

Table 1

Sample ID/GenBank number	Species	Sample ID/GenBank number	Species
KY084480	* Afarsia avinovi	GQ128970	Itylos titicaca
KY084479	* Afarsia avinovi dangara	JX093488	Kretania alcedo
FJ663565	Afarsia iris	JX093457	Kretania eurypilus
JX093487	Afarsia morgiana	JX093458	Kretania eurypilus zamotajlovi
AY556936	Afarsia morgiana	GQ128990	Kretania pylaon
VL02X393	Afarsia morgiana	JX093464	Kretania zephyrinus
HM404831	* Afarsia morgiana badgiri	AY556955	Lysandra albicans
HM404832	* Afarsia morgiana badgiri	JX093472	Lysandra bellargus
HM404833	* Afarsia morgiana badgiri	AY557132	Lysandra caelestissima
HM404834	* Afarsia morgiana badgiri	AY557055	Lysandra coridonius
HM404835	* Afarsia morgiana badgiri	AY557133	Lysandra gennargenti
HM404846	* Afarsia morgiana seleucus	AY556993	Lysandra ossmar
HM404847	* Afarsia morgiana seleucus	AY557010	Lysandra syriaca
FJ663567	<u>Afarsia rutilans</u>	JX093485	Maurus vogelii
FJ663569	Afarsia sieversi haberhaueri	GQ128985	Nabokovia cuzquenha
FJ663570	Afarsia sieversi haberhaueri	GQ128986	Nabokovia faga
FJ663571	Afarsia sieversi haberhaueri	AY496811	Neolysandra alexander
GQ128944	Agriades dardanus	JX093490	Neolysandra coelestina
GQ128942	Agriades glandon glandon	JX093479	Neolysandra diana
GQ129011	Agriades optilete optilete	JX093469	Pamira chryopis
GQ129012	Agriades optilete yukona	GQ128987	Paralycaeides inconspicua
GQ128945	Agriades orbitulus	JX093475	Patricius lucifer
JX093466	Agriades pheretiades	HM404862	Plebejidea afshar
GQ128943	Agriades podarce	GQ128989	Plebejidea loewii
JA093400	Alpherakya sarta	JA093408	Plebejus argus
FJ003250	<u>Alpherakya sarta</u>	JA093407	Plebejus argyrognomon
FJ003238	<u>Alpherakya sartolaes</u>	GQ128974	Plebejus calilopis armoricanelius
IX002482	Aricia aegestis	GQ128973	Plebejus taas atrapraetextus
JA093482 JX002476	Aricia akinongia	GQ128973	Plebuling emigdionis
JA093470 JX002450	Aricia chinensis	E1662226	Piebuina emigaionis Polyommatus gatinidas
GO128002	Aricia visias	AV556086	Polyommatus admatus
IX003480	Aricia vandarbani	AV556017	Polyommatus aadon
FI663704	Chilades galba	IX093452	Polyommatus amandus
GO128946	Chilades laius	IX093486	Polyommatus cornelia
IX093458	Cvaniris semiargus	AY496852	Polyommatus dagmara
GU688448	Cvaniris semiargus	AY557121	Polyommatus damon
GO128953	Eumedonia eumedon	AY496735	Polyommatus damone
FI663554	Eumedonia persephatta	IX093461	Polyommatus daphnis
GO128956	Freveria nuti	AY556992	Polyommatus darvlas
GO128955	Freveria trohilus	KY111670	*Polyommatus elbursicus
IX093489	Glabroculus cvane	IX093470	Polyommatus erotides
FJ663577	Glabroculus cvane	KY111669	*Polyommatus hamadanensis
JX093456	Glabroculus elvira	AY556994	Polyommatus icarus
FJ663579	Glabroculus elvira	KT983470	Polyommatus iphigenia
GQ128960	Hemiargus hanno	KT582701	Polyommatus karindus
GQ128957	Hemiargus hanno bogotanus	KT261288	Polvommatus kendevani
GQ128958	Hemiargus hanno gyas	EF104619	Polyommatus magnificus
GQ128959	Hemiargus hanno gyas	AY557001	Polyommatus menalcas
GQ128949	Hemiargus hantingtoni	AY496851	Polyommatus poseidonides
GQ128950	Hemiargus martha	KT983472	Polyommatus shirkuhensis
GQ128961	Hemiargus ramon	KT261282	Polyommatus shahkuhensis
GQ128962	Icaricia acmon	AY556959	Polyommatus thersites
GQ128963	Icaricia icarioides	JX093493	Rimisia miris
GQ128966	Icaricia saepiolus	FJ664026	Rimisia miris
GQ128967	Icaricia Shasta	JX093477	Rueckbelia fergana
GQ128978	Itylos huascarana	GQ129009	**Talicada nyseus
GQ128979	Itylos koa	GQ129010	**Tongeia fischeri
GQ128969	Itylos mashenka	GQ128947	**Cupido minimus
GQ128983	Itylos sigal	GQ128954	**Cupido comyntas
GQ128984	Itylos tintarrona	JX093474	**Leptotes trigemmatus

List of studied material (122 specimens). (*) – sequences obtained in the present study. (**) – sequences used as an outgroup



Fig. 2. The phylogenetic tree of the subtribe Polyommatina based on analysis of the *cytochrome oxidase subunit I (COI)* gene from 122 samples. Numbers at nodes indicate MP bootstrap values/ ML bootstrap values/ Bayesian posterior probability respectively with non-matching clades using different analyses indicated by '-'. Posterior probability (for Bayesian inference) and bootstrap values (for MP and MP analyses) >50% are shown. Representatives from the subtribes Cupidina and Leptotina were used as outgroups. The position of *P. avinovi* individuals indicated by an asterisk (*).

A fragment of this tree demonstrating the position of the target species *P. avinovi* and closely related taxa is shown in Fig. 3. Analysis of mitochondrial marker *COI* has shown the nominal *P. avinovi* and *P. avinovi dangara* to be highly homogeneous genetically.

In the original descriptions *P. avinovi* and *P. avinovi* dangara (STSHETKIN 1980; ECKWEILER 1997) were treated as a part of the genus *Polyommatus*, close to *P. magnificus*. Molecular data does not

support this hypothesis and demonstrates that *P. avinovi* is a taxon phylogenetically very distant from *P. magnificus* and from the genus *Polyommatus* as a whole. The morphological analysis made by ZHDANKO (2004), showed that the male genitalia structure of *P. avinovi* is generally similar to those found in *Rimisia*. Thus, *P.avinovi* was transferred by Zhdanko to this genus (ZHDANKO 2004). However, our phylogenetic reconstruction shows that *P. avinovi* is not closely related to *Rimisia* and represents a highly differentiated and



Fig. 3. Fragment of consensus phylogenetic tree of the subtribe Polyommatina inferred from *COI* sequences showing detailed clustering of *Polyommatus avinovi* and closely related species. The position of the target species *Polyommatus avinovi* is highlighted. bootstrap values/ ML bootstrap values/ Bayesian posterior probability respectively with non-matching clades using different analyses indicated by '-'. Posterior probability (for Bayesian inference) and bootstrap values (for MP and MP analyses) >50% are shown.

strongly supported monophyletic lineage that cannot possibly be subsumed within Rimisia as it would result in a paraphyletic assemblage. The studied specimens of P. avinovi form a clade that is sister to the genus Afarsia. The representatives of the genus Afarsia have an enlarged round black spot on the wing underside, usually centered by a few metallic scales (TUZOV et al. 2000; ZHDANKO 2004). This enlarged round black spot can be seen on the wing underside of Polyommatus avinovi (indicated by arrows in the Fig. 1a, b), however in two studied samples the metallic scales are absent. In P. magnificus this spot is not enlarged, not round and not darker than other spots of the marginal row (Fig. 1c, indicated by arrows). Generally, the wing underside in P. avinovi strongly resembles the wing underside in Afarsia sieversi haberhaueri (Staudinger, 1886) (Fig. 1e). Genetic divergence of the P. avinovi individuals as compared to species of the Afarsia genus is moderately low (3.6-5.8%), suggesting that *P. avinovi* should be included within Afarsia.

In conclusion, we have dealt with the case when a particular species cannot be attributed to the specific genus based on their morphological characters alone, because the same morphological traits are shared among different genera. This situation could be the result of unrecognized parallelisms in the evolution of some morphological characters or of the long preservation of plesiomorphic states that are mistakenly considered synapomorphies, or of both processes acting simultaneously in different characters (TALAVERA *et al.* 2013a). Such taxonomic and identification problems can be solved if a combination of morphological and molecular markers is applied.

Combining the morphological and DNA barcode data as suggested by LUKHTANOV *et al.* (2016), we create a new taxonomic hypothesis and propose the following new combinations for this butterfly: *Afarsia avinovi* comb. nov. and *Afarsia avinovi dangara* comb. nov. Thus, our work clarifies the taxonomic position and status of *P. avinovi*, which was considered in the literature either as a species of *Polyommatus* or *Rimisia*, but actually belongs to *Afarsia*.

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